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Identification of Distinct Thiopeptide-Antibiotic Precursor Lead Compounds Using Translation Machinery Assays

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SUMMARY

Most thiopeptide antibiotics target the translational machinery: thiostrepton (ThS) and nosiheptide (NoS) target the ribosome and inhibit translation factor function, whereas GE2270A/T binds to the elongation factor EF-Tu and prevents ternary complex formation. We have used several in vitro translational machinery assays to screen a library of thiopeptide antibiotic precursor compounds and identified four families of precursor compounds that are either themselves inhibitory or are able to relieve the inhibitory effects of ThS, NoS, or GE2270T. Some of these precursors represent distinct compounds with respect to their ability to bind to ribosomes. The results not only provide insight into the mechanism of action of thiopeptide compounds but also demonstrate the potential of such assays for identifying lead compounds that might be missed using conventional inhibitory screening protocols.

INTRODUCTION

The translational machinery represents one of the major targets within the cell for antibiotics (reviewed by Spahn and Prescott, 1996; Wilson, 2004). Many clinical important classes of antibiotics, such as the tetracyclines, phenylpropanoids (chloramphenicol), macrolides (erythromycin), and aminoglycosides (gentamicin), inhibit translation by binding to the ribosome. Despite the potency of many of these drug classes, antibiotic resistance among clinically relevant pathogens is an increasing problem and thus the need for new antibiotics is more urgent than ever before. One class of antibiotics that has received renewed interest in recent years is the thiopeptide family (reviewed by Bagley et al., 2005; Nicolaou et al., 2009) because of their effectiveness against Gram-positive bacteria, in particular, methicillin-resistant *Staphlococcus aureus*, as well as against the malarial parasite *Plasmodium falciparum* (McConkey et al., 1997). Thiopeptide antibiotics are composed of oxazoles and thiazoles, as well as non-natural amino acids that are linked together to form complex macrocyclic frameworks (Figures 1A–1D).

Two distinct families of thiopeptide compounds target the translational apparatus, one that targets the ribosome, referred to here as Class I thiopeptides, and the other, referred to as Class II thiopeptides, which targets the elongation factor EF-Tu. The best characterized of the Class I compounds include thiostrepton (ThS) and nosiheptide (NoS) (Figures 1A and 1B), both of which have been crystallized bound to the large ribosomal subunit (Harms et al., 2008) (see later Figures 6A, 6B, and 6G). These structures reveal that the Class I thiopeptides bind within a region of the ribosome that is part of the GTPase-associated center, so-named because it is involved in translation G protein factor binding and stimulation of GTPase activity (Wilson and Nierhaus, 2005). Biochemically, Class I thiopeptides have been shown to inhibit 70S initiation complex (70SIC) formation by interfering with the initiation G protein IF2 (Brandi et al., 2004, and references therein; Grigoriadou et al., 2007) as well as elongation by interfering with both the G proteins EF-Tu (Gale et al., 1981; Gonzalez et al., 2007; Modolell et al., 1971), which is necessary for rapid cognate aminoacyl-tRNA binding to the ribosome, and EF-G, which catalyzes translocation of the $tRNA_2$-mRNA complex from the A and P sites to the P and E sites (Pan et al., 2007; Pestka, 1970; Rodnina et al., 1999; Seo et al., 2006; Weisblum and Demohn, 1970). In contrast, the structurally similar Class II thiopeptides (Figure 1D) do not bind to the ribosome, but instead interact directly with EF-Tu (reviewed by Parmeggiani and Nissen, 2006). The crystal structure of the Class II thiopeptide GE2270A bound to EF-Tu reveals that the drug binds within a cleft between domains I and II of EF-Tu and directly overlaps with the binding site of the terminal end of the aminoacyl-tRNA (Parmeggiani et al., 2006; Parmeggiani and Nissen, 2006) (see later Figures 7A–7C). GE2270A is thought to prevent the closing of domain I and II, which is necessary for the induced-fit binding of EF-Tu to the tRNA, thereby preventing ternary complex formation (Parmeggiani and Nissen, 2006).

Although ThS is already in veterinary usage, its low water solubility and poor bioavailability has so far precluded its use in

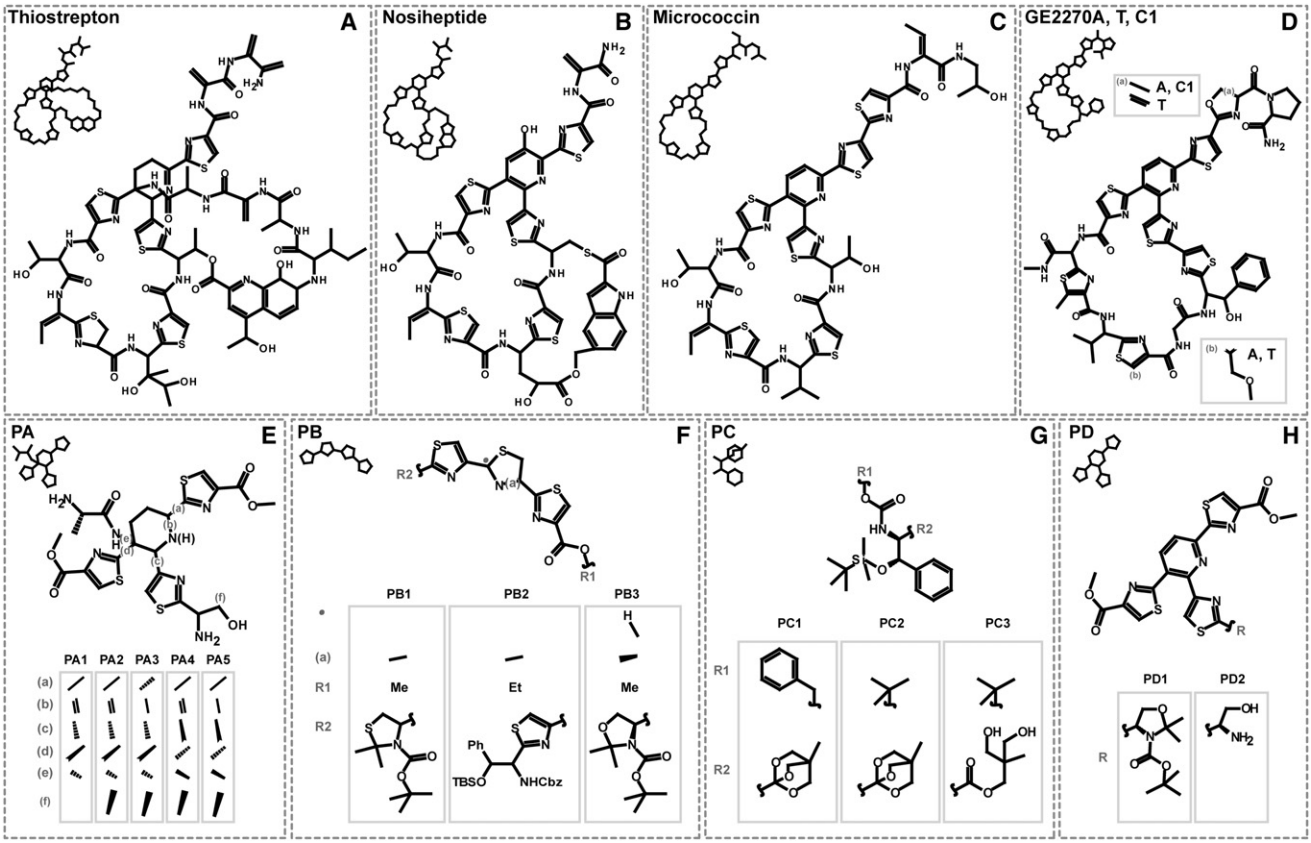

Figure 1. Chemical Structures of Thiopeptide Antibiotics and Precursor Families PA–PD

Chemical structures of the thiopeptide antibiotics ThS (A), NoS (B), micrococcin (MiC) (C), and GE2270A/T/C1 (D) and precursor families PA1-5 (E), PB1-3 (F), PC1-3 (G), and PD1-2 (H).

human medicine. Recent success has been reported in the total synthesis of a number of Class I and II thiopeptides (reviewed by Hughes and Moody, 2007; Nicolaou et al., 2009), including among others ThS (Nicolaou et al., 2005a, 2005c) and GE2270A (Nicolaou et al., 2006, 2008b). Such synthetic studies pave the way to generating improved thiopeptide derivatives by identifying synthetic fragments (or derivatives thereof) that display biological activity or can act as new lead compounds.

We have used a series of translation machinery assays to screen a library of thiopeptide antibiotic precursor compounds. Unlike the parent antibiotics ThS, NoS, and GE2270T, only a few of the precursor compounds display any significant inhibitory properties, even at high concentrations. Instead, however, four structurally distinct families of precursor compounds (Figures 1E–1H) were discovered that relieve the inhibitory effect imparted by the parent compounds. The different precursor families exhibit differential effects with respect to the inhibitory antibiotic that is counteracted as well as to the target, whether it is the ribosome or EF-Tu. Two of the families represent completely new compounds with respect to their ability to bind to ribosomes and thus open the path to the development of novel antimicrobials. The application of such screening strategies will enable the identification of new lead compounds that are not detected using conventional inhibitory screening protocols.

RESULTS

Several assays were used to examine thiopeptide precursor compounds for their abilities to bind to the ThS binding site, either by mimicking ThS inhibition of specific ribosomal functions or by protecting the ribosome against the inhibitory effects of ThS via a competition effect. These assays, which are discussed in turn below, measure (1) IF2 conformational change during the conversion of 30S initiation complex (30SIC) to 70SIC, (2) ribosome-dependent stimulation of the GTPase activity of EF-G, and (3) the cell-free synthesis of green fluorescent protein (GFP) using an *Escherichia coli* in vitro-coupled transcription-translation (TT) assay.

The Thiopeptide Precursor PA1 Inhibits 70SIC Formation

The initiation factor IF2 is essential for 70SIC formation from 30SIC and 50S subunits (Antoun et al., 2003; Grigoriadou et al., 2007). Elsewhere we have shown that the fluorescence of a Cy3 derivative of IF2 ($IF2^{Cy3}$) increases on 70SIC formation resulting from the binding of a 50S subunit to a 30SIC-containing $IF2^{Cy3}$ (Qin et al., 2009). This increase is inhibited by both ThS and NoS (Figure 2A), largely as a result of the effect of these antibiotics in inhibiting both the rate and extent of 70SIC formation (Grigoriadou et al., 2007; data not shown). Measuring the extent

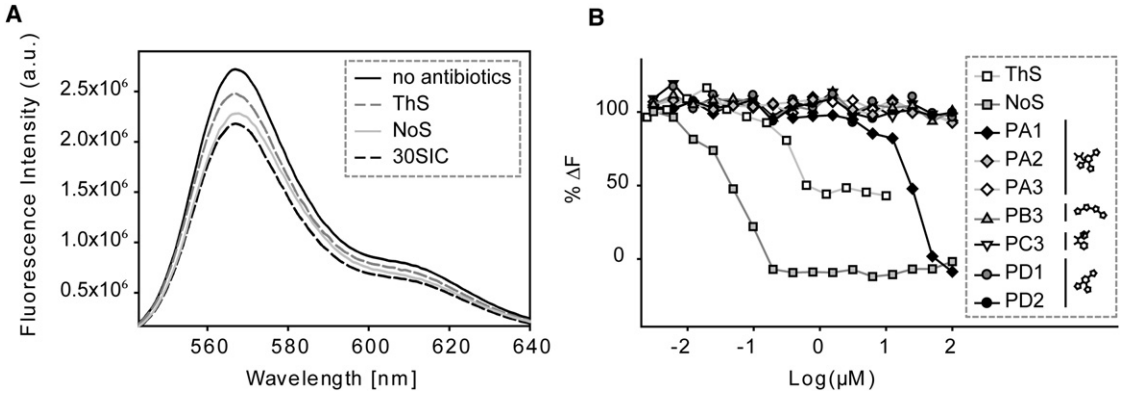

Figure 2. The IF2 Fluorescence Change Assay

(A) Emission spectra of $IF2^{Cy3}$-containing 30SIC mixed with 50S subunits in the presence of ThS or NoS. 50S was preincubated with antibiotics for 5 min at 37°C and then rapidly mixed with 30SIC at 20°C, followed by 5 min incubation before measurements. Black solid trace, no antibiotics; gray dashed trace, ThS; gray solid trace, NoS; black dashed trace, 30SIC alone. The final concentrations are IF1, IF3, and $fMet\text{-}tRNA^{fMet}$, 0.45 μM; $IF2^{Cy3}$, 0.15 μM; mRNA, 0.90 μM; 30S, 0.30 μM; 50S, 0.30 μM; GTP, 100 μM; ThS or NoS, 1.5 μM.

(B) Dose-response curves for the inhibition of fluorescence change on mixing $IF2^{Cy3}$-containing 30SIC with 50S subunits in the presence of thiopeptide compounds. The y axis indicates the percent ΔF in the presence of added compound relative to the ΔF in the absence of added compound relative to the fluorescence of 70SIC by itself. Final concentrations are $IF2^{Cy3}$, 0.15 μM; IF1, IF3, and $fMet\text{-}tRNA^{fMet}$, 0.45 μM; 022AUGmRNA, 0.9 μM; 30S, 0.30 μM; 50S, 0.30 μM; GTP, 100 μM.

of fluorescence change is thus a convenient way of monitoring thiopeptide precursor effects on 70SIC formation. A library of thiopeptide precursor compounds, as well three forms of the EF-Tu inhibitor GE2270 (A, T, and C1), were screened for this activity, along with ThS and NoS as positive controls. Only one precursor, denoted PA1 (Figure 2B), showed any measurable activity in inhibiting the fluorescence change, with an apparent K_i of 25 μM, some 60- to 400-fold higher than for ThS or NoS, respectively. Although PA1 does not bind with very high affinity, it apparently does so with considerable structural specificity, since the inhibitory effect was not seen for compounds PA2 and PA3, which have either stereochemical or minor constitutional differences from PA1 (Figure 1E). The thiopeptide precursors were also screened for their abilities to reverse ThS inhibition of the $IF2^{Cy3}$ fluorescence increase on 70SIC formation. In no case was such reversal observed.

Differential Effects of Precursor Compounds on Factor-Dependent GTPase Assays

Vacant 70S ribosomes are known to stimulate the GTPase activity of EF-G via formation of a 70S•EF-G•GTP complex. Such stimulation is strongly inhibited by ThS (Pestka, 1970; Weisblum and Demohn, 1970). We used two multiple turnover GTPase assays to screen thiopeptide precursors for inhibitory activity.

The first assay measured EF-G GTPase activity via formation of a Malachite Green complex (see Experimental Procedures) for hundreds of turnovers. Under conditions for which ThS (1 μM) almost completely abolished such activity, we identified three distinct classes of precursor compounds (PA–PC; Figures 1E–1G) that exhibited modest inhibitory effects when added at 50 μM (Figure 3A). However, we note that none of these compounds added at 10 μM showed appreciable inhibition (data not shown). As expected, the negative control, GE2270T, had no effect at a concentration of 50 μM (Figure 3A). In order to determine whether the modest inhibitory effects seen in Figure 3A were specific for EF-G, we next checked whether these compounds could also inhibit the ribosome-dependent stimulation of the Tet(M) GTPase. Tet(M) is a GTPase that binds to the ribosome analogously to EF-G and confers resistance to the antibiotic tetracycline by weakening its binding to the ribosome (reviewed by Connell et al., 2003). Similar to the results for EF-G, representatives of the PA and PC families exhibited modest inhibitory effects on Tet(M) GTPase at high concentration (100 μM) (Figure 3B). However, in contrast to EF-G, little or no inhibition was observed for the PB family, suggesting that it is specific for EF-G.

The second assay measured EF-G GTPase activity from the fluorescence increase of released Pi binding to the fluorescent phosphate binding protein MDCC-PBP (Brune et al., 1994; Seo et al., 2006). This assay, which can in principle be used for single turnover measurement, was here used to measure several turnovers, as determined by the stoichiometric ratio (5:1) of MDCC-PBP to ribosome. As performed, this assay could only detect very potent inhibitors of EF-G GTPase, since fluorescence was not measured until 1 min after initiation of reaction, whereas in the absence of inhibition the full fluorescence change is complete within 5–10 s (data not shown). It is thus no surprise, given the results presented in Figure 3A, that although ThS inhibited this assay with an apparent K_i of 1.1 μM none of the precursors tested, nor even NoS or MiC, showed measurable inhibition up to a concentration of 100 μM (Figure 3C). In contrast, both NoS and MiC added at very low concentration protected against inhibition by 1.2 μM ThS, with half-maximal effects seen at 0.04 μM and 0.11 μM, respectively (Figure 3D). However, none of the precursor compounds afforded similar protective effects up to 50–100 μM of added precursor.

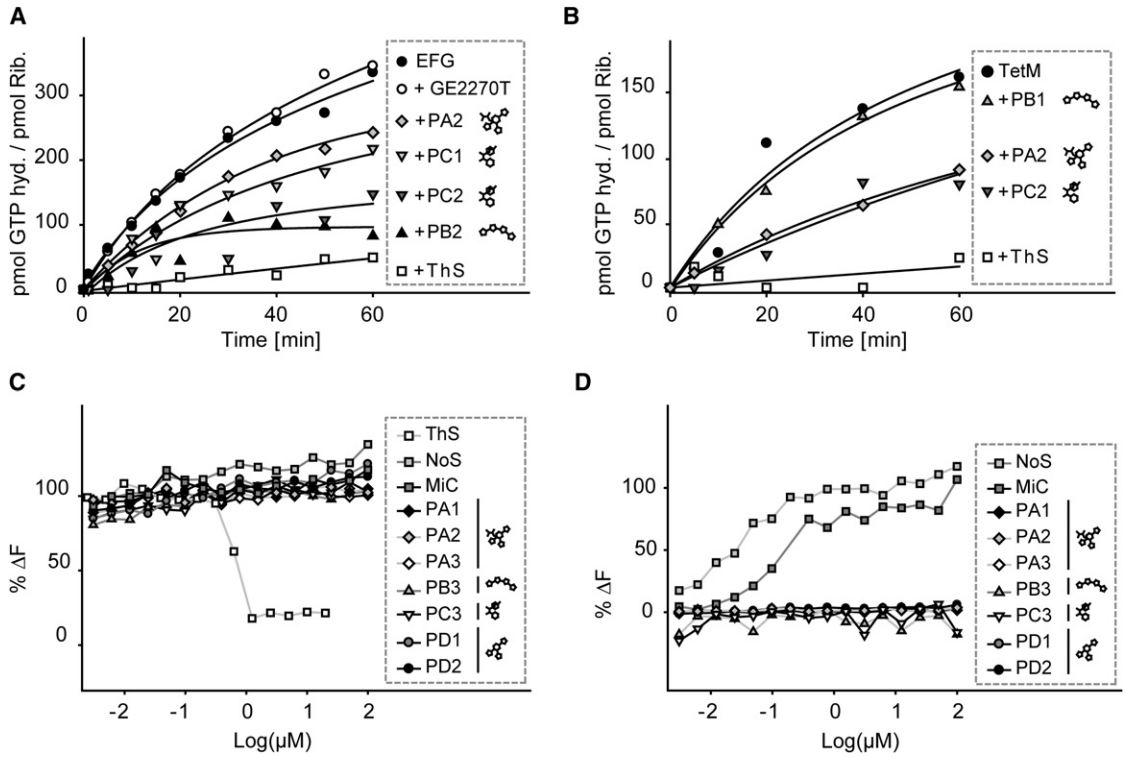

Figure 3. Effect of Thiopeptides and Precursor Compounds on GTPase Activity of EF-G and Tet(M)

(A) Inhibition of uncoupled ribosome-dependent Tet(M) GTPase by ThS (1 μM) and precursors PA2, PB2, and PC2 (50 μM). Closed circles indicate GTPase activity of Tet(M) in the absence of antibiotic.

(B) Inhibition of uncoupled ribosome-dependent TetM GTPase by ThS (1 μM) and precursors PA2, PB1, and PC2 (100 μM). Closed circles indicate GTPase activity of TetM in the absence of antibiotic.

(C) The dose-response curves of Pi release in the presence of ThS, NoS, MiC, or precursor compounds. The y axis indicates the percent ΔF due to Pi release in the presence of added compound relative to the ΔF in the absence of added compound relative to the fluorescence from EF-G interaction with the ribosome in the absence of any compound. The final concentrations are 70S, 0.3 μM; EF-G, 0.75 μM; MDCC-PBP, 1.5 μM; GTP, 100 μM;7-methylguanine, 200 μM; nucleotide phosphorylase, 0.3 U/ml.

(D) Dose-response curves for reversal of ThS inhibition of Pi release by NoS, MiC, or precursor compounds. The final concentrations for each component are 70S, 0.3 μM; EF-G, 0.75 μM; ThS, 1.2 μM; MDCC-PBP, 1.5 μM; GTP, 100 μM; 7-methylguanine, 200 μM; nucleotide phosphorylase, 0.3 U/ml.

Protective Effects of Precursor Compounds on Thiopeptide-Mediated Translation Inhibition

Although, as expected, ThS, NoS, and GE2270T were potent inhibitors of GFP synthesis using an in vitro TT assay (Figure 4, lanes 2–4), none of the precursor compounds tested displayed any significant inhibitory activity in this assay (Figure 4, white bars), even at high concentrations (50–100 μM). In contrast, addition of 50 μM of representative precursor compounds from four structural distinct classes PA–PD (Figures 1E–1G) could reverse the inhibitory effect of 5 μM ThS (Figure 4, gray bars). The most effective protection was seen with PA2, which restored translation back to levels observed in the absence of antibiotic (Figure 4, lane 6). In comparison, PB1, PC1, and PD1 restored translation to 40%–60% of the original levels (Figure 4, lanes 13–28). Additionally, we find that the 5S, 6R stereoisomer of PA2 (PA4) exhibited some protective properties (~35% compared to 100% for PA2). The structural specificity of these effects is clear from the failure of precursors that are chemically related to PA2 to exhibit similar protective effects against ThS inhibition. These include PA3 (Figure 4, lanes 6 and 8), which differs from PA2 by lacking only a double bond within the central dehydropiperidine ring (Figure 1E), and PD2 (Figure 4, lanes 26 and 28), which, with respect to PD1, has an altered side chain on one of the thiazole rings (Figure 1H).

Interestingly, PA2, PB1, PC1, and PD1 displayed marked differences in their abilities to reverse the inhibitory effects of NoS (5 μM) and GE2270T (25 μM), as compared to the inhibitory effects of ThS. Thus, as shown in Figure 5, PA2 was an omnipotent protector of translation, restoring translation levels in the presence of all three thiopeptide inhibitors, with the following order of efficiency: ThS (100%) > GE2270T (80%) > NoS (60%). PD1 rescued translation in the presence of ThS and GE2270T, but not NoS, and PB1 and PC1 efficiently rescued translation only against ThS. As was true for ThS, neither PA3, PB2, PC2, nor PD2 were able to reverse inhibition by NoS or GE2270T (data not shown).

Interaction of Thiopeptide Precursors with the Ribosome

The specific protective effect of the precursor compounds against ThS suggests that these compounds specifically compete with ThS for binding to the ribosome. Structural (Harms

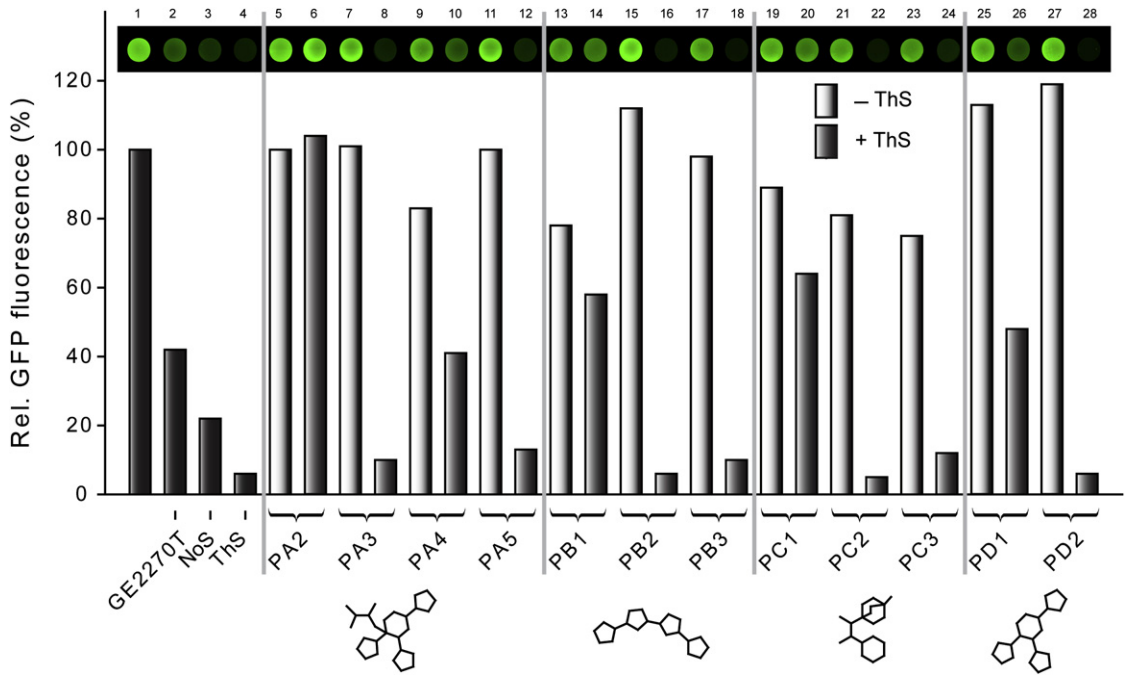

Figure 4. Precursor Compounds Protect Translation from ThS Inhibition

In vitro TT of GFP in the absence or presence of 25 μM GE2270T, 5 μM NoS, and 5 μM ThS (black bars) or in the presence (50 μM) of precursor families PA2-5, PB1-3, PC1-3, and PD1-2 (−ThS; white bars) alone or with additional presence of 5 μM ThS (+ThS; gray bars). GFP fluorescence from microtiter plate wells shown above each lane were quantified and represented as bars, with the fluorescence detected in the absence of antibiotic assigned as 100%.

et al., 2008; Jonker et al., 2007) and biochemical data (Spahn and Prescott, 1996; Xing and Draper, 1996) for ThS suggests that the high affinity of this drug for the ribosome results from cooperative interaction between nucleotides in H43/44 of the 23S rRNA and the L11-NTD (Figures 6A and 6B). Given the structural similarity between PA2 and ThS, it is possible to model the position of this compound bound to the ribosome (Figure 6C; Harms et al., 2008). The substitution of the double bond in the piperidine ring of PA2 to generate PA3 abolishes the protective effect of the compound (Figure 4). This is likely to result from differences in the planarity of the piperidine ring between PA3 and PA2, which in turn leads to differences in the relative position (by 0.5 Å) of the attached thiazole moiety, which, based on the model, would shift it toward Pro27 of L11-NTD and thus encroach on the thiopeptide binding site (data not shown). Such modest displacements within drug binding sites have been shown to have dramatic effects on the affinity of compounds and often lead to antibiotic resistance (Blaha et al., 2008; Tu et al., 2005).

Although it is more difficult to model the PB and PC series of compounds based on the available structures, it is clear that the aromatic rings within these families suggest a potential mode of binding that establishes simultaneous stacking interactions with both H43/44 and L11-NTD (Figures 6D and 6E). Alterations that disrupt these rings, as seen for PC2 (Figure 6F), could explain a reduced binding and corresponding loss in protection (Figure 4). The PD class of precursors is structurally most similar to the pyridine core of NoS (Figure 1). NoS is oriented differently on the ribosome compared to ThS, establishing stacking interactions with Pro22 but not Pro26 (Figure 6G; Harms et al., 2008). Based on our modeling, PD1 can make analogous interactions with Pro22 as NoS (Figure 6H), whereas the inactive PD2 cannot (Figure 6I).

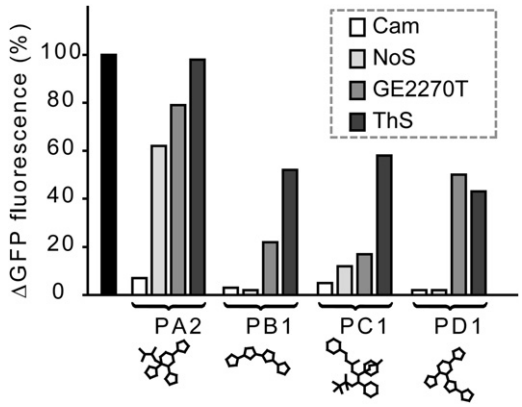

Figure 5. Differential Protective Effects of Precursors Against Thiopeptide Inhibition

Protection profiles of representative precursors from all described groups against chloramphenicol (Cam; 10 μM), NoS (5 μM), GE2270T (25 μM), and ThS (5 μM). GFP fluorescence in the absence of antibiotic is assigned as 100%, whereas the precursor results are presented as the percentage of protection, given as the difference between the inhibition of translation by the active compound (Cam, NoS, GE2270T, or ThS) in the presence and absence of the precursor compound (50 μM).

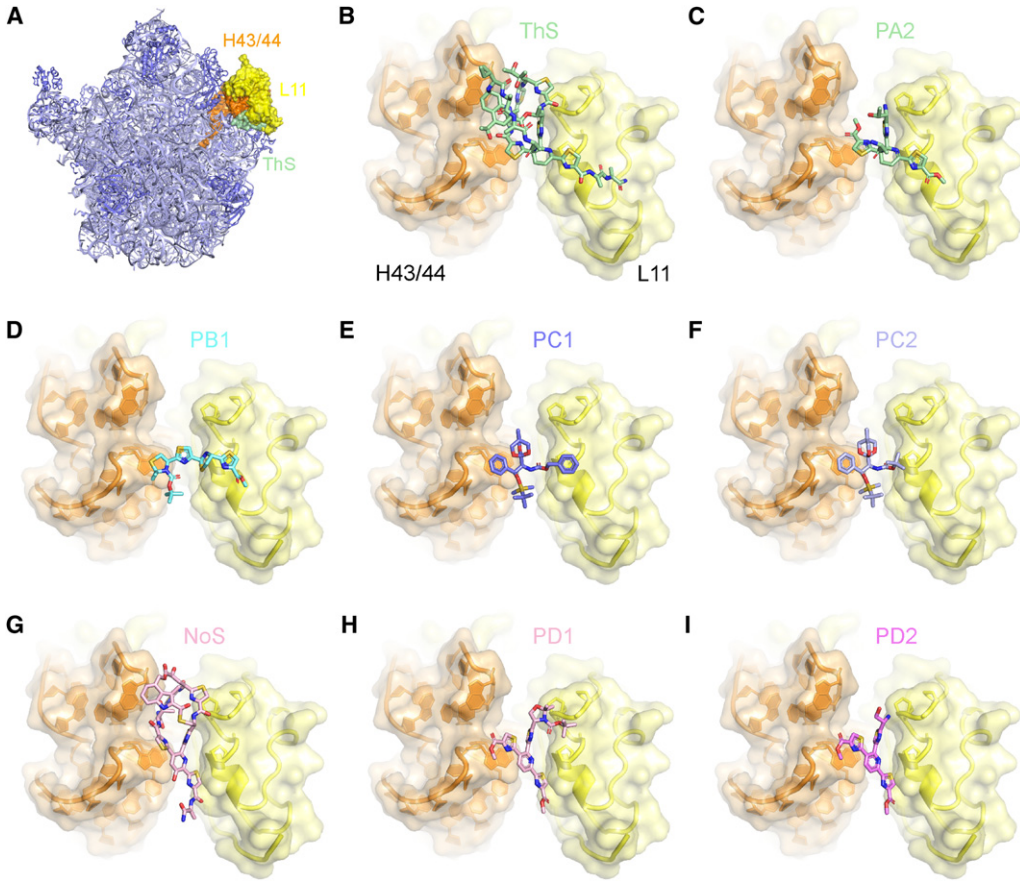

Figure 6. Binding Site of Precursor Compounds on the Ribosome

(A) Overview of thiopeptide binding site on the large ribosomal subunit. Interface view with helix 43 and 44 (H43/44; orange), L11 (yellow), and ThS (green) highlighted with surface representation (from PDB ID 3CF5) (Harms et al., 2008).

(B) The thiazole rings of ThS (green) interact with the RNA bases at the tips of H43/44 as well as the prolines in the N-terminal domain of L11 (yellow).

(C) Model for precursor PA2 bound to the ribosome, based on the position of ThS. PA1, but not PA3 (see text), bind similarly.

(D–F) Possible modes of binding for precursors PB1 and PC1 based on ring stacking interactions with RNA and protein components of the ribosome, whereas PC2 lacks one phenyl ring compared to PC1.

(G) NoS (pink) interacts with the RNA bases at the tips of H43/44 as well as the N-terminal domain of L11 (yellow), but in a distinct manner compared to ThS (using PDB ID 2ZJP) (Harms et al., 2008).

(H and I) Model for precursor PD1 and PD2 bound to the ribosome, based on the position of NoS. PD2 lacks one ring moiety, suggesting binding would be destabilized.

Interaction of Thiopeptide Precursors with EF-Tu

The thiopeptide GE2270A has been crystallized in complex with EF-Tu, revealing that the drug binds within a covered groove in domain II and spans across the active site cleft of EF-Tu, the G domain, to interact with the domain I (Figure 7A; Parmeggiani et al., 2006). GE2270A overlaps the binding site of the terminal A76 and aminoacyl moiety of the tRNA (Figure 7B) and is believed to prevent the closing of domains I and II necessary for the induced fit binding of aa-tRNA (Figure 7C), thereby preventing ternary complex (EF-Tu•GTP•tRNA) formation (reviewed by Parmeggiani and Nissen, 2006). The structural similarities between PA and PD and GE2270A (Figure 1) suggest that these compounds would also bind within the groove of domain II of EF-Tu (Figure 7D) and overlap with the A76 of the tRNA (Figure 7E). However, the truncated nature of these compounds prevents them from establishing additional interactions with domain I, even in the closed tRNA-bound ternary complex state of EF-Tu (Figure 7F).

DISCUSSION

Development of improved antimicrobial agents will be necessary to combat the prevalence of multi-drug-resistant bacteria. A number of biochemical approaches have been taken to identify functionally important hotspots on the ribosome that do not overlap with previously known antibiotic binding sites (Laios et al., 2004; Llano-Sotelo et al., 2009b; Yassin et al., 2005; Yassin and Mankin, 2007). In addition, a recent study has developed an assay using fluorescently labeled ribosomal proteins to monitor binding of small molecules, such as antibiotics, to the ribosome, which is amenable to high-throughput screening (Llano-Sotelo et al., 2009a). Here we present several translation-related assays

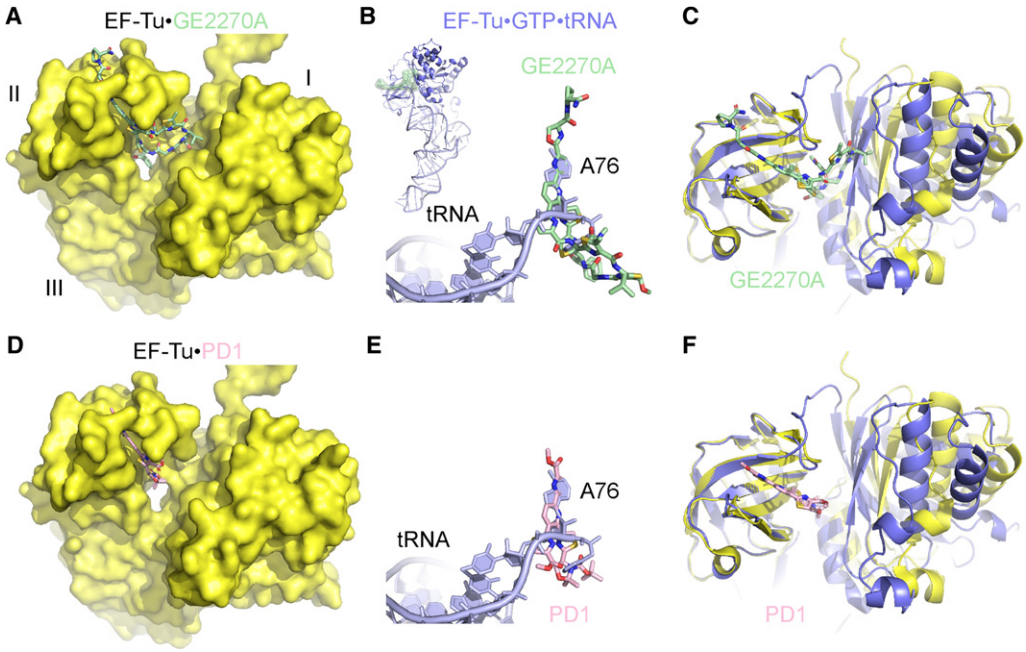

Figure 7. Binding Site of Precursor Compounds on EF-Tu

(A) Structure of the thiopeptide GE2270A (green) bound to EF-Tu (yellow), with domains I, II, and III indicated (PDB ID 2C77) (Parmeggiani et al., 2006).

(B) GE2270A overlaps the binding position on EF-Tu of the terminal A76 and aminoacyl moiety of tRNA. Inset shows overview of EF-Tu•tRNA ternary complex (PDB ID 1TTT) (Nissen et al., 1995) with superimposition of GE2270A.

(C) Superimposition of EF-Tu•GE2270A (yellow) and EF-Tu•tRNA (blue) aligned on basis of domain II. Note that GE2270A (green) clashes with domain I of EF-Tu from the ternary complex (blue).

(D) Model for precursor PD1 bound to EF-Tu based on EF-Tu•GE2270A complex (PDB ID 2C77) (Parmeggiani et al., 2006).

(E) PD1 (pink) overlaps the binding position on EF-Tu of the terminal A76 and aminoacyl moiety of tRNA (blue).

(F) As (C) but with PD1 instead of GE2270A. Note that PD1 does not clash with domain I of EF-Tu from the ternary complex (blue).

using high-throughput 96 or 384 microtiter plate formats that have been used to screen a library of thiopeptide precursor compounds for their abilities to inhibit one or more aspects of translation and/or reverse the inhibition of known thiopeptide antibiotics. These screens identified four distinct families of precursor compounds, termed PA–PD, which could act as potential lead compounds for development of novel antimicrobials.

Two of the families identified, PA and PD, contain a six-membered nitrogen heterocycle core (PA, dehydropiperidine; PD, pyridine) analogous to the thiopeptide antibiotics ThS and GE2270A (Figure 1). The crystal structures of thiopeptides bound to the ribosome (Harms et al., 2008) and of GE2270A bound to EF-Tu (Parmeggiani et al., 2006) reveal the importance of the heterocycle core of these compounds for interaction with their respective targets and allows modeling of how PA and PD members are likely to interact with the ribosome and/or EF-Tu (Figures 5 and 6). The resulting models are consistent with the rescue of translation in the presence of ThS and GE2270T by family members, such as PA2 and PD2, probably by direct competition for binding between the precursor compound and the thiopeptide antibiotic. In addition, PA1 and PA2 displayed inhibitory activity against translational GTPases IF2 (Figure 2B) and EF-G and Tet(M) (Figures 3A and 3B), respectively. However, compared to the parent thiopeptide compounds, much higher concentrations of the precursor compounds were necessary to exhibit similar effects, most likely indicating the much lower binding affinity of the precursors. The ineffectiveness of precursor compound PA2 as a direct inhibitor was surprising, since this compound has been previously reported to exhibit antimicrobial activity against methicillin-resistant *Staphlococcus aureus* and vancomycin-resistant *Enterococcus faecalis* with a minimal inhibitory concentration (MIC) of 5 μM (Nicolaou et al., 2005b). Our results suggest therefore that the inhibitory effect of PA2 in vivo may in fact not be related to translation, but verification of this point will require further investigation.

The other two families identified in our screen, PB and PC, have not, to our knowledge, been previously reported to target the translational machinery. PB1 is chemically similar to the thiazolidine precursor compound used to generate the pyridine core of amythiamicins (Nicolaou et al., 2008a), which target EF-Tu analogously to GE2270A (Parmeggiani et al., 2006; Parmeggiani and Nissen, 2006). The PC series of compounds contain a protected β-hydroxy-α-aminoacid, which is a precursor in the synthesis of GE2270A/T/C1. Curiously, the PB and PC families display much higher specificity for the ribosome than for EF-Tu, as shown by the ability of PB1 and PC1 to restore translation more efficiently in the presence of ThS, as compared with GE2270A (Figure 5). Although PB1 and PC1 are structurally distinct (Figure 1), we believe the common aromatic/cyclic nature of both these compounds is important for ribosome binding. Accommodation of EF-G on the ribosome involves the

insertion of domain V of EF-G into the crevice between H43/44 and L11-NTD. Inhibition by Class I thiopeptides has been proposed to stem in part from their physically linking L11-NTD to H43/44, thereby locking the cleft shut (Harms et al., 2008). We suggest that PB1 and PC1 can also span the L11-rRNA crevice (Figures 6D and 6E) and perform this locking function, analogous to ThS/PA2 (Figures 6B and 6C) and NoS/PD1 (Figures 6G and 6H). Similarly to PA/D, the high concentrations of PB/C required to inhibit the ribosome-dependent GTPase activity of EF-G are indicative of their low binding affinities for the ribosome. Such low affinity may allow facile displacement of precursors from the ribosome, as a result of translation factors (IF2 or EF-G) binding, or from EF-Tu, during ternary complex formation, thus explaining the absence of any direct inhibitory effect of any of the precursors on GFP synthesis. The differential effects of the precursors on the GTPase assays compared to the TT assay is probably related to the ribosome concentrations in the GTPase assays being ~10×–100× less (30–300 nM) compared to the TT assay (~2 μM) and to the putative higher affinity of EF-G for translating rather than empty ribosomes (Sergiev et al., 2005).

The majority of clinically used antibiotics targeting the ribosome bind either to the decoding region on the small subunit or within either the peptidyltransferase center or the adjacent peptide exit tunnel of the large subunit, where they interact almost exclusively with ribosomal RNA (Spahn and Prescott, 1996; Wilson, 2004). The Class I thiopeptide compounds, however, are distinct in that they target a different region of the ribosome, namely the GTPase-associated region or translation factor binding site, where they interact with both rRNA and ribosomal protein L11. As a consequence, no cross-resistance has been found between thiopeptide antibiotics and other clinically important drugs. The compounds such as PA–PD identified in our study provide lead structures for the development of novel antimicrobial agents that target this region of the ribosome. Furthermore, the ability of some precursor compounds, such as PA1 and PD1, to bind both EF-Tu and the ribosome suggests the feasibility of developing antimicrobials that are dual inhibitors of ribosomes and ternary complex formation.

SIGNIFICANCE

The translational machinery represents one of the major targets within the cell for antibiotics, with many clinical important classes of antibiotics inhibiting translation by binding to the ribosome. Despite the potency of many of these drug classes, antibiotic resistance among clinically relevant pathogens is an increasing problem and there is an urgent need for improved antibiotics. We present herein a series of translation machinery assays that can be used to screen for lead compounds that not only inhibit specific steps of translation but also relieve the inhibitory effects of other inhibitory compounds. Using these assays to screen a library of thiopeptide precursor compounds, we have identified four distinct families of compounds that inhibit either IF2, EF-G, and/or Tet(M), as well as confer protective effects against thiopeptide translation inhibitors of both the ribosome and EF-Tu. Our findings not only elucidate the mechanism of action of thiopeptide compounds but also illustrate the potential of such high-throughput assays to identify distinct lead compounds that might be missed using conventional inhibitory screening protocols. Whereas the IF2 and EF-G GTPase assays are specifically useful for screening antibiotics interfering with translation G factor proteins, the TT assay is generally applicable for screening all classes of translation inhibitors, including those targeting the peptidyltransferase, and decoding centers of the ribosome and other ribosomal sites, in addition to those interfering with translation G factor proteins.

EXPERIMENTAL PROCEDURES

Component Preparation

GE2270A, T, and C1 and the library of thiopeptide precursor compounds were synthesized as described previously (Nicolaou et al., 2005b, 2006, 2008a, 2008b). ThS was purchased from Sigma, whereas NoS was a gift from H.G. Floss and micrococcin P1 was supplied by T. Stachlhaus. The *tetM* gene (Tn916) cloned into the pET24b vector was a gift from V. Burdett. Tet(M) protein was expressed in BL21 (DE3) pRIL cells in 20°C with 0.2 mM IPTG. *E. coli* EF-G cloned into pQE70 vector was expressed in XL1 blue cells. Both proteins were purified using Ni-NTA metal affinity chromatography (QIAGEN), followed by gel filtration chromatography on a HiLoad 26/60 Superdex 75 prep grade column (GE Healthcare). For the experiments described in Figures 2 and 3C and 3D, ribosomes, $IF2^{Cy3}$, IF1, IF3, 30S subunits, MDCC-labeled phosphate-binding protein (MDCC-PBP), 022AUG mRNA, and $fMet\text{-}tRNA^{fMet}$ were prepared as described previously (Qin et al., 2009) as was EF-G (Pan et al., 2007).

$IF2^{Cy3}$ Fluorescence Change Assay

Reactions were carried out in a 384 well assay plate. 50S subunits were preincubated (15 min at 37°C) with a series of concentrations of the test compounds in DMSO that are transferred from a premade compound plate to the assay plate by a PerkinElmer Evolution P3 liquid handler. Reaction was initiated by addition of 30SIC to each well of the plate. Fluorescence (579 nm) was read with a 2103 EnVision Multilabel Plate Reader on excitation at 550 nm. For the reversal experiment, 30SIC was preincubated with ThS (10 min at 37°C), followed by a second preincubation with test compounds (10 min at 37°C), and reaction was initiated by 50S addition.

GTPase Activity Assays

For both assays described below, reactions performed in the absence of ribosomes were used as a background signal to account for the intrinsic GTPase activity of EF-G or Tet(M).

By Malachite Green

GTPase activity was measured using the Malachite Green Phosphate Kit (BioAssay) that quantifies the green complex formed between Malachite Green, molybdate, and free orthophosphate. All reactions contained 30 nM *E. coli* 70S ribosomes, 20 μM GTP, and 60 nM protein in the presence or absence of antibiotics as necessary. Reactions were transferred into 96 well microtiter plates and color formation was measured on Tecan Infinite M1000 microplate reader at 650 nm.

By MDCC-Labeled PBP

GTPase activity was measured using the MDCC-labeled PBP complex, which measures free phosphate release as an increase in fluorescence and uses a Pi-MOP system to minimize the background due to phosphate present in the original medium (Brune et al., 1994; Seo et al., 2006). Reactions were carried out in a 384 well assay plate. Ribosomes were preincubated (15 min at 37°C) with a series of concentrations of the test compounds in DMSO that are transferred from a premade compound plate to the assay plate by a PerkinElmer Evolution P3 liquid handler. Reaction was initiated by addition of an ice-cold solution containing EF-G, MDCC-PBP, and GTP to each well of the plate, a process that was completed in under 30 s. Fluorescence (450 nm) was read within 1 min using a 2103 EnVision Multilabel Plate Reader, on excitation at 405 nm. For the reversal experiment, ribosomes were preincubated with ThS (10 min at 37°C), followed by a second preincubation with test

compounds (10 min at 37°C), and reaction was initiated by EF-G, MDCC-PBP, and GTP as above.

In Vitro Transcription-Translation Assay

All coupled TT experiments were performed using an *E. coli* lysate-based system in the presence and absence of antibiotics as described previously (Dinos et al., 2004; Szaflarski et al., 2008). Reactions were transferred into 96 well microtiter plates and the GFP fluorescence was measured with a Typhoon Scanner 9400 (GE Healthcare) using a Typhoon blue laser module (GE Healthcare). Images were then quantified using ImageQuantTL (GE Healthcare) and represented graphically using SigmaPlot (Systat Software, Inc.).

Modeling and Figure Preparation

Chemical structures for the precursor compounds were drawn and converted to 3D coordinates using ChemDraw (Advanced Chemistry Development, Inc.). PA2 models used the ThS binding position on the *Deinococcus radiodurans* 50S (D50S) subunit (PDB ID 3CF5) (Harms et al., 2008), whereas PD1 and PD2 were based on the D50S-NoS complex (PDB ID 2ZJP) (Harms et al., 2008). PyMol (http://www.pymol.org) was used to model the PB1 and PC compounds, align EF-Tu•GE2270A (yellow; PDB ID 2C77) (Parmeggiani et al., 2006) and EF-Tu•tRNA (blue; PDB ID 1TTT) (Nissen et al., 1995) on the basis of domain II, as well as to prepare all X-ray structure figures.

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REFERENCES

Antoun, A., Pavlov, M.Y., Andersson, K., Tenson, T., and Ehrenberg, M. (2003). The roles of initiation factor 2 and guanosine triphosphate in initiation of protein synthesis. EMBO J. *22*, 5593–5601.

Bagley, M.C., Dale, J.W., Merritt, E.A., and Xiong, X. (2005). Thiopeptide antibiotics. Chem. Rev. *105*, 685–714.

Blaha, G., Gurel, G., Schroeder, S.J., Moore, P.B., and Steitz, T.A. (2008). Mutations outside the anisomycin-binding site can make ribosomes drug-resistant. J. Mol. Biol. *379*, 505–519.

Brandi, L., Marzi, S., Fabbretti, A., Fleischer, C., Hill, W., Lodmell, J., and Gualerzi, C. (2004). The translation initiation functions of IF2: targets for thiostrepton inhibition. J. Mol. Biol. *335*, 881–894.

Brune, M., Hunter, J.L., Corrie, J.E., and Webb, M.R. (1994). Direct, real-time measurement of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment 1 ATPase. Biochemistry *33*, 8262–8271.

Connell, S.R., Tracz, D.M., Nierhaus, K.H., and Taylor, D.E. (2003). Ribosomal protection proteins and their mechanism of tetracycline resistance. Antimicrob. Agents Chemother. *47*, 3675–3681.

Dinos, G., Wilson, D.N., Teraoka, Y., Szaflarski, W., Fucini, P., Kalpaxis, D., and Nierhaus, K.H. (2004). Dissecting the ribosomal inhibition mechanisms of edeine and pactamycin: the universally conserved residues G693 and C795 regulate P-site tRNA binding. Mol. Cell *13*, 113–124.

Gale, E.F., Cundliffe, E., Reynolds, P.E., Richmond, M.H., and Waring, M.J. (1981). Antibiotic inhibitors of ribosome function. In The Molecular Basis of Antibiotic Action, E.F. Gale, E. Cundliffe, P.E. Reynolds, M.H. Richmond, and M.J. Waring, eds. (Bristol, UK: John Wiley and sons), pp. 278–379.

Gonzalez, R.L., Jr., Chu, S., and Puglisi, J.D. (2007). Thiostrepton inhibition of tRNA delivery to the ribosome. RNA *13*, 2091–2097.

Grigoriadou, C., Marzi, S., Kirillov, S., Gualerzi, C.O., and Cooperman, B.S. (2007). A quantitative kinetic scheme for 70 S translation initiation complex formation. J. Mol. Biol. *373*, 562–572.

Harms, J.M., Wilson, D.N., Schluenzen, F., Connell, S.R., Stachelhaus, T., Zaborowska, Z., Spahn, C.M., and Fucini, P. (2008). Translational regulation via L11: molecular switches on the ribosome turned on and off by thiostrepton and micrococcin. Mol. Cell *30*, 26–38.

Hughes, R.A., and Moody, C.J. (2007). From amino acids to heteroaromatics—thiopeptide antibiotics, nature's heterocyclic peptides. Angew. Chem. Int. Ed. Engl. *46*, 7930–7954.

Jonker, H.R., Ilin, S., Grimm, S.K., Wohnert, J., and Schwalbe, H. (2007). L11 domain rearrangement upon binding to RNA and thiostrepton studied by NMR spectroscopy. Nucleic Acids Res. *35*, 441–454.

Laios, E., Waddington, M., Saraiya, A.A., Baker, K.A., O'Connor, E., Pamarathy, D., and Cunningham, P.R. (2004). Combinatorial genetic technology for the development of new anti-infectives. Arch. Pathol. Lab. Med. *128*, 1351–1359.

Llano-Sotelo, B., Hickerson, R.P., Lancaster, L., Noller, H.F., and Mankin, A.S. (2009a). Fluorescently labeled ribosomes as a tool for analyzing antibiotic binding. RNA *15*, 1597–1604.

Llano-Sotelo, B., Klepacki, D., and Mankin, A.S. (2009b). Selection of small peptides, inhibitors of translation. J. Mol. Biol. *391*, 813–819.

McConkey, G.A., Rogers, M.J., and McCutchan, T.F. (1997). Inhibition of Plasmodium falciparum protein synthesis. Targeting the plastid-like organelle with thiostrepton. J. Biol. Chem. *272*, 2046–2049.

Modolell, J., Cabrer, B., Parmeggiani, A., and Vazquez, D. (1971). Inhibition by siomycin and thiostrepton of both aminoacyl-tRNA and factor G binding to ribosomes. Proc. Natl. Acad. Sci. USA *68*, 1796–1800.

Nicolaou, K.C., Safina, B.S., Zak, M., Lee, S.H., Nevalainen, M., Bella, M., Estrada, A.A., Funke, C., Zécri, F.J., and Bulat, S. (2005a). Total synthesis of thiostrepton. Retrosynthetic analysis and construction of key building blocks. J. Am. Chem. Soc. *127*, 11159–11175.

Nicolaou, K.C., Zak, M., Rahimipour, S., Estrada, A.A., Lee, S.H., O'Brate, A., Giannakakou, P., and Ghadiri, M.R. (2005b). Discovery of a biologically active thiostrepton fragment. J. Am. Chem. Soc. *127*, 15042–15044.

Nicolaou, K.C., Zak, M., Safina, B.S., Estrada, A.A., Lee, S.H., and Nevalainen, M. (2005c). Total synthesis of thiostrepton. Assembly of key building blocks and completion of the synthesis. J. Am. Chem. Soc. *127*, 11176–11183.

Nicolaou, K.C., Zou, B., Dethe, D.H., Li, D.B., and Chen, D.Y. (2006). Total synthesis of antibiotics GE2270A and GE2270T. Angew. Chem. Int. Ed. Engl. *45*, 7786–7792.

Nicolaou, K.C., Dethe, D.H., and Chen, D.Y. (2008a). Total syntheses of amythiamicins A, B and C. Chem. Commun. (Camb.) 2632–2634.

Nicolaou, K.C., Dethe, D.H., Leung, G.Y., Zou, B., and Chen, D.Y. (2008b). Total synthesis of thiopeptide antibiotics GE2270A, GE2270T, and GE2270C1. Chem. Asian J. *3*, 413–429.

Nicolaou, K.C., Chen, J.S., Edmonds, D.J., and Estrada, A.A. (2009). Recent advances in the chemistry and biology of naturally occurring antibiotics. Angew. Chem. Int. Ed. Engl. *48*, 660–719.

Nissen, P., Kjeldgaard, M., Thirup, S., Polekhina, G., Reshetnikova, L., Clark, B.F., and Nyborg, J. (1995). Crystal structure of the ternary complex of Phe-tRNAPhe, EF-Tu, and a GTP analog. Science *270*, 1464–1472.

Pan, D., Kirillov, S.V., and Cooperman, B.S. (2007). Kinetically competent intermediates in the translocation step of protein synthesis. Mol. Cell *25*, 519–529.

Parmeggiani, A., and Nissen, P. (2006). Elongation factor Tu-targeted antibiotics: four different structures, two mechanisms of action. FEBS Lett. *580*, 4576–4581.

Parmeggiani, A., Krab, I.M., Okamura, S., Nielsen, R.C., Nyborg, J., and Nissen, P. (2006). Structural basis of the action of pulvomycin and GE2270 A on elongation factor Tu. Biochemistry *45*, 6846–6857.

Pestka, S. (1970). Thiostrepton: a ribosomal inhibitor of translocation. Biochem. Biophys. Res. Commun. *40*, 667–674.

Qin, H., Grigoriadou, C., and Cooperman, B.S. (2009). Interaction of IF2 with the ribosomal GTPase-associated center during 70S initiation complex formation. Biochemistry *48*, 4699–4706.

Rodnina, M.V., Savelsbergh, A., Matassova, N.B., Katunin, V.I., Semenkov, Y.P., and Wintermeyer, W. (1999). Thiostrepton inhibits the turnover but not the GTPase of elongation factor G on the ribosome. Proc. Natl. Acad. Sci. USA *96*, 9586–9590.

Seo, H.S., Abedin, S., Kamp, D., Wilson, D.N., Nierhaus, K.H., and Cooperman, B.S. (2006). EF-G-dependent GTPase on the ribosome. Conformational change and fusidic acid inhibition. Biochemistry *45*, 2504–2514.

Sergiev, P.V., Lesnyak, D.V., Kiparisov, S.V., Burakovsky, D.E., Leonov, A.A., Bogdanov, A.A., Brimacombe, R., and Dontsova, O.A. (2005). Function of the ribosomal E-site: a mutagenesis study. Nucleic Acids Res. *33*, 6048–6056.

Spahn, C.M.T., and Prescott, C.D. (1996). Throwing a spanner in the works: antibiotics and the translational apparatus. J. Mol. Med. *74*, 423–439.

Szaflarski, W., Vesper, O., Teraoka, Y., Plitta, B., Wilson, D.N., and Nierhaus, K.H. (2008). New features of the ribosome and ribosomal inhibitors: non-enzymatic recycling, misreading and back-translocation. J. Mol. Biol. *380*, 193–205.

Tu, D., Blaha, G., Moore, P., and Steitz, T. (2005). Structures of MLSBK antibiotics bound to mutated large ribosomal subunits provide a structural explanation for resistance. Cell *121*, 257–270.

Weisblum, B., and Demohn, V. (1970). Inhibition by thiostrepton of the formation of a ribosome-bound guanine nucleotide complex. FEBS Lett. *11*, 149–152.

Wilson, D.N. (2004). Antibiotics and the inhibition of ribosome function. In Protein Synthesis and Ribosome Structure, K. Nierhaus and D. Wilson, eds. (Weinheim: Wiley-VCH), pp. 449–527.

Wilson, D.N., and Nierhaus, K.H. (2005). Ribosomal proteins in the spotlight. Crit. Rev. Biochem. Mol. Biol. *40*, 243–267.

Xing, Y., and Draper, D.E. (1996). Cooperative interactions of RNA and thiostrepton antibiotic with two domains of ribosomal protein L11. Biochemistry *35*, 1581–1588.

Yassin, A., and Mankin, A.S. (2007). Potential new antibiotic sites in the ribosome revealed by deleterious mutations in RNA of the large ribosomal subunit. J. Biol. Chem. *282*, 24329–24342.

Yassin, A., Fredrick, K., and Mankin, A.S. (2005). Deleterious mutations in small subunit ribosomal RNA identify functional sites and potential targets for antibiotics. Proc. Natl. Acad. Sci. USA *102*, 16620–16625.

